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[RAPID COMMUNICATION]

Immunocytochemical Localization of Troponin I and C in the Muscles of *Caenorhabditis elegans*

Hiroki Nakae[1] and Takashi Obinata[2]

[1]*Advanced Research Laboratory, Research and Development Center, Toshiba Corporation 1, Komukai Toshiba-cho, Saiwai-ku, Kawasaki 210, and* [2]*Department of Biology, Faculty of Science, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263, Japan*

ABSTRACT—Troponin-I (TNI) and troponin-C (TNC) in muscle tissues of a nematode, *C. elegans*, were investigated using antibodies against the respective troponin-components of *Ascaris*. Immunoblot analysis demonstrated that these antibodies recognize the troponin components of *C. elegans* that have the same molecular masses as the corresponding troponin components in *Ascaris* body-wall muscle. In the immunocytochemical staining of the serial cryosections of *C. elegans*, the anti-TNI antibody stained both the pharyngeal and the body-wall muscles. On the other hand, the anit-TNC antibody reacted with the body-wall muscle but not with the pharyngeal muscle. These results suggest that variants of troponin components exist between the pharyngeal and body-wall muscles, and troponin is involved in the actin-linked regulatory system of *C. elegans*.

INTRODUCTION

Caenorhabditis elegans is an eligible system for studying morphogenesis of muscle. Various protein components in the *C. elegans* muscles have been identified and investigated genetically and biochemically. Especially, contractile proteins, for example, actin, myosin, and paramyosin, have been studied intensively (for review, [19]). The existence of actin- and myosin-linked calcium regulatory systems for muscle contraction was suggested in *C. elegans* [8]. The myosin regulatory light chains of 18 kDa which may be responsible for the myosin-linked regulation have been characterized (for review, [1]). However, only limited attempts have so far been made to clarify the actin-linked Ca^{2+}-regulatory proteins in *C. elegans*.

Troponin, a Ca^{2+}-dependent regulatory protein, is localized on actin filaments and plays a significant role together with tropomyosin in the Ca^{2+}-dependent regulation of muscle contraction [3, 4]. In the previous study, troponin composed of three components, namely troponin-T (TNT), troponin-I (TNI), and troponin-C (TNC), was detected in the muscle of a nematode, *Ascaris lumbricoides*, and the components were isolated and characterized [9]. Although it is known that some invertebrate troponin, for example in ascidian [5] and molluscan [16] muscles, activates actin-myosin interaction in a Ca^{2+}-dependent manner, the *Ascaris* troponin inhibited actomyosin ATPase in the absence of Ca^{2+}, but did not activate it in the presence of Ca^{2+}. Moreover, the polyclonal antibodies against TNI and TNC were prepared by immunizing rabbits with each troponin component and location of troponin components along this filaments at constant periodicity was demonstrated by immunoelectron microscopy [9]. In this study, the existence of the troponin components in *C. elegans* has been ascertained,

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[1] To whom correspondence should be addressed.

and their immunocytochemical localization has been investigated, using the cross-reactivity of the antibodies against *Ascaris* troponin components.

MATERIALS AND METHODS

Caenorhabditis elegans ver. Brisol (strain N2) was grown at 20°C on NG agar plates with *Escherichia coli* OP50 as feed, described by Brenner [2].

The affinity-purified polyclonal antibodies against *Ascaris* TNI and TNC were prepared as described [9]. The antibodies were absorbed with acetone-dried liver powder. Fluorescein (FITC)-labeled goat anti-rabbit IgG antibody (GAR) was purchased from Cappel Laboratories.

For all studies with nematodes, worms on agar plates were washed in M9 buffer (6.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 5.0 g NaCl and 0.25 g $MgSO_4 \cdot 7H_2O$ in 1 l H_2O), and collected by incubating at 4°C for 1 hr. For SDS-polyacrylamide gel electrophoresis (PAGE), an SDS-sample buffer (2% SDS, 2 mM β-mercaptoethanol, 40 mM sodium-phosphate buffer, pH 7.0) was added to the pellet of worms, and then the mixture was frozen in liquid nitrogen. The frozen mixture was dissolved immediately by incubating at 95°C for 3 min, and applied for SDS-PAGE. SDS-PAGE was carried out using 13.5% polyacrylamide gel according to Laemmli [10]. Peptides were transferred electrophoretically from electrophoretic gel to nitrocellulose paper according to Towbin *et al.* [18], and the paper was reacted with the antibodies against *Ascaris* TNI or TNC. Immunoglobulin bound to the paper was detected with ^{125}I-labelled protein A.

Squash preparation was performed basically according to Miller *et al.* [13]. A small droplet of the collected adult worms was placed between two 3% gelatin-coated glass slides, squashed by gentle pressure, and then immersed directly in liquid nitrogen until frozen. The slides were pried on dry ice with a razor blade. The slides were treated with ethanol cooled at −20°C for 10 min, and air-dried.

To prepare specimens for cryosections, the pellet of adult worms in M9 buffer was mixed with Tissue Tek-II O.C.T. compound (Miles Lab. Inc.), then immersed in liquid nitrogen-cooled isopentane. Serial cryosections were cut at 8 μm, and rapidly air-dired.

Immunostaining was performed as follows. The squashed specimens and the sections were fixed with phosphate buffered saline (PBS) containing 3.5% formalin for 5 min. After washing with PBS, the specimens were treated with PBS containing 1% bovine serum albumin. They were then exposed to the antibodies against TNI or TNC, and the antibody binding was detected with FITC-GAR. Each antibody incubation was performed for 1 hr at room temperature followed by thorough wshing in PBS. The antibody-treated specimens were mounted in 90% glycerol-10% 0.2 M carbonate buffer (pH 8.6), and the fluorescence was observed and photographed by using a fluorescence and phase contrast microscope.

RESULTS

At first, immunoblotting was performed to

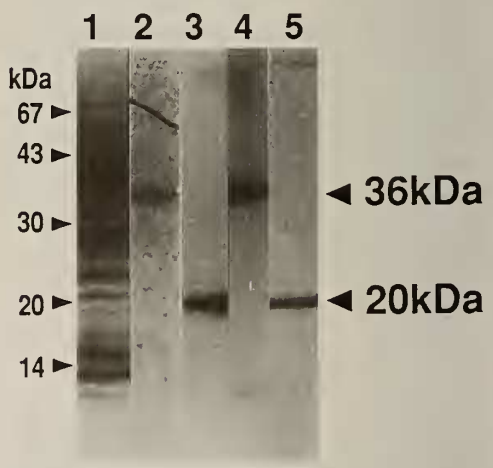

FIG. 1. Cross-reactivity of the antibodies against *Ascaris* TNI and TNC with a whole protein extract of *C. elegans*. The protein extracted with an SDS-sample buffer from *C. elegans* (1, 2, 3), purified *Ascaris* TNC (4), TNC (5) were electrophoresed on 13.5% polyacrylamide gels. The extract was transferred on nitrocellulose paper, and then reacted with the anti-TNI (2) or the anti-TNC (3) antibodies. Lane 1 shows the Amide black stainign of the extract on the nitrocellulose paper. The anti-TNI and anti-TNC antibodies reacted with the protein bands having approximately same molecular masses to *Ascaris* TNI (36 kDa) and TNC (20 kDa), respectively.

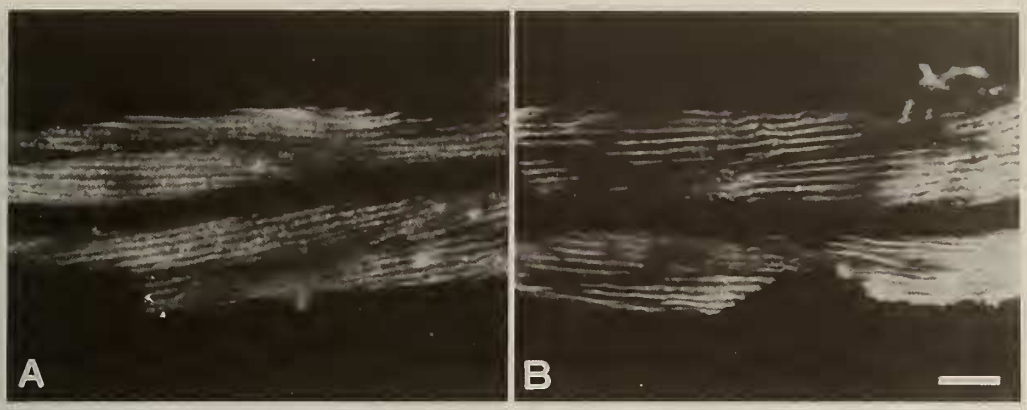

FIG. 2. Indirect immunofluorescent stainig of squashed specimens of *C. elegans*. Bar: 10 μm. The squashed specimens were treated with anti-TNI (A) and the anti-TNC (B) antibodies.

examine whether the antibodies prepared by immunizing *Ascaris* TNI and TNC cross-react with the corresponding proteins in *C. elegans*. Figure 1 shows the results of the immunoblotting. Each antibody reacted with a single peptide band in the whole extract of *C. elegans*. Moreover, the apparent molecular masses of the proteins of *C. elegans* which were recognized by the anti-*Ascaris* TNI and TNC were almost equivalent to those of the respective troponin components of *Ascaris* which were described previously [9]. Therefore, it is reasonable to conclude that the antibodies against *Ascaris* TNI and TNC also recognize TNI and TNC in *C. elegans,* respectively.

Using the antibodies, the immunocytochemical localizations of TNI and TNC in *C. elegans* were examined by staining the squashed specimens (Fig. 2) and the serial cross-sections in the head region (Fig. 3). As shown in Figure 2, antibodies stained the squashed specimens of the animal to give striated patterns. The positively stained regions probably correspond to I-band of myogibrils in body-wall muscle, because the spots of reduced staining, which seemed to correspond to the positions of dense-bodies, were observed in the striation of the staining. Further, anti-TNI and TNC exhibit different reactivity to the muscles of nematode. The sections shown in Figure 3 contained two kinds of muscles namely body-wall and pharyngeal muscles. Body-wall muscles were stained positively with both antibodies. On the other hand, pharyngeal muscle exhibited the different reactivity to anti-TNI or anti-TNC antibodies. As shown in Figure 3 anti-TNI antibody stained the pharyngeal muscle clearly, while anti-TNC antibody failed to react with this muscle.

DISCUSSION

The immunoblot analyses showed that the antibodies which were prepared against the *Ascaris* TNI and TNC, recognize the protein bands of *C. elegans,* whose molecular masses are equivalent to TNI and TNC in *Ascaris* muscles. It has been also ascertained by immunocytochemical methods that the respective antigens are localized in the I-bands of the body-wall muscles of *C. elegans*. Considering these results together with the close phylogenetic relationships between *Ascaris* and *C. elegans,* we have concluded that the troponin regulatory system functions in *C. elegans* muscles as in *Ascaris,* and both animals contain troponin components of identical size. In this study, we could not examine the other troponin component, TNT, but it is likely that TNT also exists in the muscle of *C. elegans*.

We further observed that the pharyngeal muscle and the body-wall muscle exhibit different immunoreactivity to the anti-troponin antibodies. The anti-TNI antibody exhibited positive reaction with both body-wall and pharyngeal muscles, while anti-TNC antibody reacted only with body-wall muscle. These results may potentiate three interpretations. First, pharyngeal muscle may not have

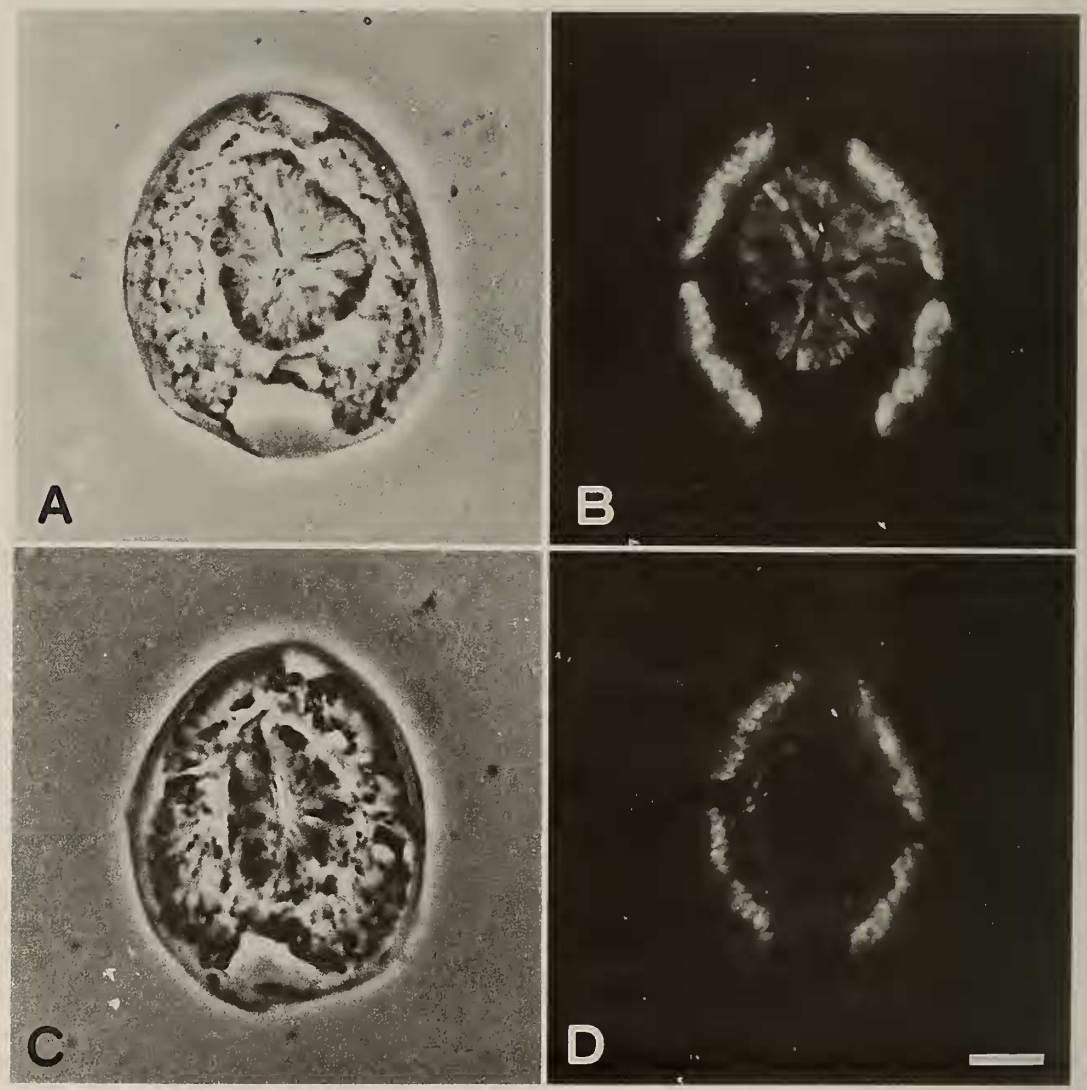

FIG. 3. Immunocytochemical localization of the TNI and TNC in the muscles of *C. elegans* head region. Bar: 10 μm. Serial cross sections of worms were treated with anti-TNI (A, B) and the anti-TNC (C, D) antibodies. Florescent images (B, D) were corresponded to the phase-contrast micrographs (A, C).

TNC. Secondly, the TNC in pharyngeal muscle may be too small in amount to be detected. Thirdly, pharyngeal muscle may have the other TNC isoform(s) with different antigenicity. Previous comparative biochemical studies have demonstrated that three troponin components mostly exist roughly in equimolar ratio in various invertebrate animals [5, 12, 16], although they vary in size and sometimes exist in multiple isoforms [15]. Therefore, it is most likely that body-wall and pharyngeal muscles of nematodes contain different TNC isoforms, although further investigation is needed to reach a definit conclusion. We can not eliminate the possibility that TNIs in the two muscles share common antigenic site(s) but differ somehow in moecular structure.

Besides the troponin conponents, it is konwn that pharyngeal muscle differs from body-wall muscle in several points. Differences in myosin isoforms between these muscles are particularly

remarkable. It has been demonstrated that four myosin heavy chain isoforms, named myoA, myoB, myoC and myoD, are distinguishable electrophoretically in the wild-type *C. elegans,* and the pharyngeal muscle possesses myoC and myoD, while the boyd-wall muscle contains myoA and myoD [6, 14, 17, 20]. Obliquely striated myofibrils have been observed in the body-wall muscle [7], but the pharyngeal muscle exhibits different structural organization [for reivew, 1]. The pharyngeal muscle seems to be functionally distinct somewhat from the body-wall muscle. Previous studies suggested that nematode muscle may be controlled dually by actin-linked and myosin-linked regulatory systems [8, 11]. Contractile activity of the two muscles of the nematode could be altered by the difference in myosin and troponin isoforms.

In conclusion, we have demonstrated that the body-wall muscle contain the troponin components, TNI and TNC, of approximately the same size as the counterparts of *Ascaris.* Thus, it is obvious that the contraction of body-wall muscle is under the control of the actin-linked troponin regulatory system. In addition, it is very likely that pharyngeal and body-wall muscles possess distinct troponin components. It is a matter of interest for furture studies how these protein variants are derived, whether encodered by different genes or generated from a single gene.

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